One-way PCR-based mapping of a replication initiation point (RIP)

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Abstract

The *lamin B2* locus is the only mammalian origin whose replication initiation points (RIPs) have been mapped. Although this paper was published 8 years ago, no further mammalian RIP-mapping studies have been reported, largely due to technical difficulties of ligation-mediated (LM)-PCR used by the authors. Here, we report the development of a simple, one-way PCR-based protocol that allows one to accurately determine RIPs at mammalian origins. The procedure can be completed within 48 h from the time of cell lysis in the agarose gel. Nascent DNA is then isolated from the same gel after DNA is separated by alkaline gel electrophoresis. Subsequently, RIPs are determined by one-way PCR-based primer extension using labeled primers. Using this protocol, we have successfully mapped RIPs in the human *DBF4* locus. As one-way PCR is routinely used by many scientists, this protocol will provide a powerful new tool for studying DNA replication in many organisms including mammalian cells.

INTRODUCTION

Faithful replication of the entire genome once per cell cycle is critical to maintain genetic integrity. Because the origin of DNA replication (*ori*) plays a key role in this process, understanding the initiation mechanism is essential, for which mapping the replication initiation point (RIP) is the first step. Several RIPs have been mapped in unicellular organisms such as bacteria and budding yeast. However, RIP mapping in mammalian cells turned out to be very challenging, mainly due to technical difficulties. The *lamin B2* locus is thus far the only mammalian *ori* whose RIPs have been mapped¹. The authors determined RIPs at this locus using the technically demanding ligation-mediated (LM) PCR¹. Although this paper was reported in 2000, no one has reported another mammalian RIP using LM-PCR or any other methods, underscoring the need to develop more effective and user friendly RIP-mapping techniques.

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Using one-way PCR-based primer extension, Gerbi and Bielinsky successfully identified the precise transition point between continuous (leading strand) and discontinuous (lagging strand) DNA synthesis at the *Saccharomyces cerevisiae* ARS1 ori^{2–4}. The original method developed by these authors² may be divided into the following six steps:

- *Isolation of bulk genomic DNA* by cesium chloride gradient centrifugation.
- Benzoylated naphthoylated DEAE (BND) chromatography to *enrich replication intermediates*.
- *Treatment with T4 polynucleotide kinase* to phosphorylate DNA molecules lacking an RNA primer (i.e., to remove randomly nicked DNA).
- *λ -exonuclease digestion* to degrade phosphorylated DNA and enrich for RNA-primed nascent DNA, which is resistant to the enzyme.
- *Primer extension* by one-way PCR with a radioactive-labeled oligonucleotide primer to map the 5'-end of a nascent DNA strand.
- *Sequencing gel electrophoresis and detection* of the amplified primer extension products.

The authors rationalized that a RIP of the leading strand coincides with the 5' end of the shortest strand detected by the primer extension because successively larger strands are presumably generated by the ligation of Okazaki fragments to the leading strand. Since it was first reported in 1997, this RIP-mapping technique has been used to determine precise RIPs at several ori loci in *Schizosaccharomyces pombe*⁵ and the fly *Sciara cropophila*⁶. RIP mapping has also been used in archaea, such as *Pyrococcus abyssi*⁷, *Sulfolobus solfataricus*⁸ and *Haloarcula* sp.⁹. However, the original RIP-mapping protocol was not considered sensitive enough to map RIPs in mammalian cells. Therefore, LM-PCR was used to map RIP at the human *lamin B2* origin¹.

We postulated that one-way PCR-based primer extension could be directly used for mapping RIPs in mammalian oris without the need of LM-PCR, if nascent strands can be effectively harvested. We could indeed achieve this goal by bypassing several steps required for enriching short nascent DNA strands. Importantly, this stream-lined protocol did not compromise the quality and purity of the nascent DNA sample. The steps that we could omit include cesium chloride gradient centrifugation, BND chromatography, treatment with T4 polynucleotide kinase to phosphorylate DNA molecules lacking an RNA-primer and λ -exonuclease treatments. Using this new approach, we successfully mapped RIPs at the human *DBF4* locus, where we have recently identified a strong ori¹⁰. This new RIP-mapping method combines one-way PCR-based primer extension with several previously reported techniques to effectively isolate nascent DNA fragments as described below (see Table 1 for comparison of different methods).

We validated our one-way PCR-based RIP mapping using the *lamin B2* locus model (see Supplementary Fig. 2 in ref. ¹⁰; http://www.nature.com/nsmb/journal/v15/n7/supinfo/nsmb.1439_S1.html). In this validation experiment, however, we had to omit betaine for the reason discussed in the section “The use of betaine in PCR” below.

This new one-way PCR-based protocol is certainly sensitive enough to determine mammalian RIPs, even relatively weak oris such as the one at the *lamin B2* locus. Nevertheless, this mapping method may not be ideal for the detection of RIPs at extremely weak mammalian ori loci.

Finally, this new protocol can also be very useful in determining RIPs in nonmammalian organisms because it can bypass several rather tedious steps. Thus, the RIP-mapping protocol described here can greatly facilitate research in the regulation of DNA replication of many different organisms including mammalian cells.

Experimental design

Cell type/preparation—To carry out RIP mapping at the *DBF4* ori locus, we used HeLa cells as the source of nascent DNA. HeLa cells were maintained in RPMI-1640 medium containing 10% (vol/vol) fetal bovine serum (Hyclone, Logan, UT), 2.05 mM L-glutamine, 100 $\mu\text{g ml}^{-1}$ streptomycin and 100 U ml^{-1} penicillin. Although we could easily detect *DBF4* RIPs from exponentially growing HeLa cells, the results were much more pronounced when nascent DNA fragments were isolated from synchronous HeLa cells at 1 h in S phase¹⁰. This was achieved by releasing HeLa cells synchronized at the G₁/S border by a double-thymidine treatment into complete medium for 1 h as described previously¹⁰.

Emetine treatment—One of the most serious problems of determining a RIP is that ligation of Okazaki fragments to a leading strand complicates the interpretation of the true initiation point of leading strand synthesis. In yeast cells, one strategy to circumvent this problem is to compare RIPs in wild-type and DNA ligase I-deficient mutants³. Since this method is difficult to apply in mammalian cells, we and others used emetine, which inhibits new production of lagging strands without affecting leading strand synthesis^{1,11}. Although concern was raised previously regarding drug treatments and the generation of potential artifactual results², we did not observe any notable problem when we determined *DBF4* RIPs with cells maintained in the presence and absence of emetine¹⁰. However, we cannot completely rule out the potential effects of emetine on RIP-mapping results because we have not systematically compared RIP patterns of cells grown in the absence and presence of emetine.

Preparation of nascent DNA strands by alkaline gel electrophoresis—Several different approaches have been developed to isolate nascent DNA without contamination by randomly nicked DNA. Isolation of DNA molecules containing RNA primer has been a preferred method for RIP mapping². However, yielding nascent strands by this approach may not be sufficiently high because many tedious steps are required. We found that the simplest and most promising approach of isolating nascent DNA strands is: (i) to lyse cells directly in the wells of alkaline agarose gels; (ii) to separate DNA by electrophoresis under denaturing conditions and (iii) to isolate short single-stranded DNA by gel extraction¹². This procedure minimizes sample handling and has been previously employed by others to map oris in mammalian cells^{13,14}. This simple method also appears to minimize DNA damage because origin enrichment was not found with nonreplicating control DNA¹⁰. If substantial

damage occurred during nascent strand preparation, we would have observed the enrichment of origin sequence from the control DNA.

The use of betaine in PCR—Another modification we made is the use of betaine in our one-way PCR. This was particularly important for the RIP mapping at the *DBF4* locus, where G+C content is very high (~70%). Betaine has been previously shown to prevent the formation of secondary structures in template DNA and promote polymerization of GC-rich sequences¹⁵. Interestingly, adding betaine to the RIP-mapping reaction for the human *lamin B2* ori resulted in a decrease in efficiency¹⁰, perhaps because of the high A+T content in this locus. The protocol presented here is optimal for the detection of RIPs at the human *DBF4* locus where the G+C content is high. Because we had to omit betaine in the reaction for the RIP mapping at the *lamin B2* ori¹⁰, a slight adjustment for mapping protocol at different oris may be necessary.

Choice of DNA polymerases—Although Vent (exo⁻) DNA polymerase has been the polymerase of choice for RIP mapping¹⁻³, this enzyme was not ideal under our experimental conditions. The possible reason for this is that the activity of this polymerase may not be compatible with betaine. In our hands, IDPol polymerase was the best for one-way PCR-based primer extension.

PCR primer design—The primers we use for RIP mapping are at least 19-bases long with a melting temperature close to 60 °C. As ~800 bases might be the maximum length that can be efficiently amplified by IDPol polymerase in the one-way PCRs¹⁰, primer distance to the potential replication start sites should be <800 bases, preferably between 100 and 500 bases.

Choice of detection system—An important advantage of our approach is that this new protocol is designed to use nonradioactive labeling. Although radioactively labeled oligonucleotides have been the choice for primer extension reactions in previous RIP-mapping studies^{1-3,5,6}, we used digoxigenin-labeled primers, followed by immunoblotting with antidigoxigenin antibodies. We have found that this nonradioactive-labeling method is sensitive enough to detect RIPs in mammalian cells. Therefore, the entire work described in our protocol can be completed on the laboratory bench, eliminating the need for a specially shielded working area or disposing radioactive wastes.

Consideration of controls—To detect potential artifacts resulted by the reaction mix (including primers), each one-way PCR experiment should include a no-template control. In addition, a control RIP-mapping reaction should be carried out, in parallel with the main experiment, using nonreplicating DNA isolated from cells in mitosis¹⁰ or cells treated with a high concentration of replication inhibitors such as aphidicolin (Fig. 1a). This is necessary to rule out the possibility that the products observed after primer extension are resulted from amplification of randomly nicked DNA, instead of replication intermediates (i.e., leading strands).

MATERIALS

REAGENTS

- 1-kb DNA molecular weight marker (New England BioLabs, cat. no. N3232)
- 100 mM dNTP set (Invitrogen, cat. no. 10297-018)
- Acrylamide (Sigma, cat. no. A8887) ! **CAUTION** Neurotoxin and potential carcinogen. Wear gloves at all times when handling acrylamide-containing solutions.
- Agarose (Invitrogen, cat no. 15510-027)
- Alkaline phosphatase-conjugated antidigoxigenin antibodies, Fab fragments (Roche Applied Science, cat. no. 11093274910 (1093274))
- Amersham Hybond N⁺ nylon membrane (GE Healthcare Life Sciences, cat. no. RPN303B)
- Ammonium persulfate (Sigma, cat. no. A9164)
- Betaine (Sigma, cat. no. B2629)
- Blocking reagent (Roche Applied Science, cat. no. 11096176001 (1096176))
- Boric acid (Sigma, cat. no. B7901)
- Bromophenol blue (Sigma-Aldrich, cat. no. B8026)
- CDP-*Star* substrate (Roche Applied Science, cat. no. 11685627001 (1685627))
- Deionized formamide (Ambion, cat. no. AM9342) ! **CAUTION** Irritant. May be mutagenic and teratogenic.
- 5'-digoxigenin-labeled primers (Integrated DNA Technologies) ▲ **CRITICAL** Ideally, primers should have a T_m of 60 °C and be purified by HPLC.
- Disodium EDTA-2H₂O (Sigma, cat. no. E5134)
- DNA molecular weight marker V, DIG-labeled (Roche Applied Science, cat. no. 10821705001).
- Double-distilled water
- Emetine dihydrochloride hydrate (Sigma, cat. no. E2375) ! **CAUTION** Highly toxic. Emetine solutions should be handled with care and disposed of accordingly.
- Ethidium bromide (Sigma, cat. no. E7637) ! **CAUTION** Toxic and potent mutagen.
- Ficoll 400 (Fluka, cat. no. 46324)
- GIBCO 1× Trypsin-EDTA solution (Invitrogen, cat. no. 25200-056)
- Glacial acetic acid (Fisher Scientific, cat. no. A38 P212)

- Glycerol (Sigma-Aldrich, cat. no. G6279)
- IDPol DNA polymerase (ID Labs Biotechnology, cat. no. IDL007; supplied with 10× magnesium-free reaction buffer and 20 mM MgSO₄ solution)

▲ **CRITICAL** Although Vent (exo⁻) DNA polymerase is commonly used in the primer extension reactions, it was not efficient under our experimental conditions. Although we used IDPol DNA polymerase in all of our experiments, other enzymes with similar characteristics may also work.

- KCl (Sigma, cat. no. P9541)
- KH₂PO₄ (Sigma, cat. no. P9791)
- Maleic acid (Sigma-Aldrich, cat. no. M153)
- Methylene bisacrylamide (Sigma, cat. no. M7279) ! **CAUTION** Irritant and potential teratogen.
- Na₂HPO₄ (Sigma-Aldrich, cat. no. 71496)
- NaCl (Sigma, cat. no. S3014)
- NaOH pellets (Sigma, cat. no. 221465)
- QIAquick gel extraction kit (Qiagen, cat. no. 28706)
- SDS (Sigma, cat. no. L4390) ! **CAUTION** Irritant.
- TEMED (Sigma, cat. no. T7024)
- Trizma base (Sigma, cat. no. T1503)
- Tween 20 (Sigma, cat. no. P9416)
- Urea (Sigma, cat. no. U6504)
- Xylene cyanol FF (Sigma-Aldrich, cat. no. X4126)

EQUIPMENT

- 1.5-ml Polypropylene micro tubes (Sarstedt, cat. no. 72.690.200)
- 0.5-ml PCR tubes (Sarstedt, cat. no. 72.735.002)
- 10-cm cell culture dishes (Sarstedt, cat. no. 83.1802.003)
- 15-ml conical tubes (Sarstedt, cat. no. 62.554.002)
- Amersham Hyperfilm ECL (GE Healthcare Life Sciences, cat. no. 28-9068-37)
- DNA-sequencing gel electrophoresis apparatus
- Horizontal DNA electrophoresis chamber (~14 cm × 10 cm gel-casting tray)
- Microcentrifuge (Eppendorf)
- PCR machine

- Trans-Blot SD Semi-dry electrophoretic transfer cell (Bio-Rad, cat. no. 170-3940)
- UV transilluminator (Chromato-Vue TM-36)
- X-ray film developer

REAGENT SETUP

0.5 M EDTA, pH 8.0—To prepare 100 ml solution, add 18.61 g of disodium EDTA-2H₂O to ~70 ml distilled water. Adjust pH to 8.0 initially with NaOH pellets and then 10 N NaOH. Adjust the final volume to 100 ml and autoclave. The solution may be stored for several months at room temperature (~21 °C), provided that it remains free of contamination.

10 N NaOH—Dissolve 40 g of NaOH in the final volume of 100 ml distilled water. It may be stored for up to 6 months at room temperature.

10× PBS (27 mM KCl, 10 mM KH₂PO₄, 1.37M NaCl, 100 mM Na₂HPO₄, pH 7.5)—To make 1 l of solution, dissolve 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄ and 2.4 g of KH₂PO₄ in ~800 ml in distilled water. Adjust pH to 7.4–7.5, and then the final volume to 1 l with distilled water, followed by autoclaving. The buffer may be stored for several months at room temperature.

1× PBS + 10% (vol/vol) glycerol—Prepare freshly at the time of use by mixing 100 µl 10× PBS with 800 µl sterile water and 100 µl glycerol. Add 5 µl of 10% (wt/vol) bromophenol blue to serve as tracking dye.

1 M Tris-HCl, pH 8.0—To prepare 300 ml solution, dissolve 36.34 g Trizma base in ~250 ml distilled water. Adjust pH to 8.0, and the final volume to 300 ml, followed by autoclaving. It may be stored for several months at room temperature.

10%(wt/vol) SDS—Dissolve 2 g of SDS in sterile water to make the final volume of 20 ml solution. It may be stored at room temperature for several months.

5× SDS lysis buffer (50mM Tris-HCl, 5mMEDTA, 0.5%(wt/vol) SDS)—Mix 500 µl of 1M Tris-HCl, pH 8.0, 100 µl of 0.5MEDTA, pH 8.0, and 500 µl of 10% (wt/vol) SDS. Adjust the final volume to 10 ml solution with sterile water. It may be stored at 4 °C for up to 1 month.

20× TE buffer (200 mM Tris, pH 8.0, 20 mM EDTA)—To prepare 100 ml buffer, mix 76 ml distilled water with 20 ml 1 M Tris-HCl, pH 8.0, and 4 ml of 0.5 M EDTA, pH 8.0. Autoclave and store at 4 °C for up to several months.

10× DNA-loading buffer—Mix 2 g Ficoll 400, 2 ml 0.5 M EDTA, pH 8.0, 1 ml 10% (wt/vol) SDS and 250 µl 10% (wt/vol) bromophenol blue. Adjust the final volume to 10 ml solution with sterile water. Aliquot and store at –20 °C for up to several months.

1-kb DNA marker solution (50 $\mu\text{g ml}^{-1}$ 1-kb DNA ladder in 1 $\times$ TE buffer)—Mix 20 μl of 500 $\mu\text{g ml}^{-1}$ stock DNA ladder (New England BioLabs) with 10 μl 20 $\times$ TE buffer, 20 μl 10 $\times$ DNA-loading buffer and 150 μl sterile water. The diluted ladder may be stored for several months at -20°C .

‘DNA ladder mix’—Prepare a fresh batch at the time of use by mixing 40 μl of 1-kb DNA marker solution, 19 μl sterile water, 3.3 μl 1 N NaOH (the final concentration is 50mM) and 2.7 μl 0.5MEDTA, pH 8.0 (the final concentration is 20 mM).

Alkaline running buffer (50 mM NaOH, 1 mM EDTA)—Prepare a fresh batch at the time of use by mixing 993 ml of distilled water with 5 ml of 10 N NaOH and 2 ml 0.5 M EDTA, pH 8.0.

50 $\times$ TAE buffer (2 M Tris, 1 M glacial acetic acid, 50 mM EDTA, pH 8.4)—Dissolve 121 g of Trizma base, 9.31 g disodium EDTA-2H₂O and 28.6 ml glacial acetic acid, and adjust the final volume to 500 ml with sterile distilled water. The pH of the solution should normally be between 8.4 and 8.5 without further adjustment. It may be stored at room temperature for several months.

Sequencing gel-loading buffer (98% (vol/vol) formamide, 10 mM EDTA pH 8.0, 0.1% (wt/vol) xylene cyanol, 0.1% (wt/vol) bromophenol blue)—To prepare 10 ml solution, mix 9.8 ml deionized formamide, 200 μl 0.5M EDTA, pH 8.0, 0.01 g xylene cyanol and 0.01 g bromophenol blue. Store at 4°C in 1 ml aliquots up to several months.

5 $\times$ BE buffer (450 mM Tris, 450 mM boric acid, 10 mM EDTA, pH 8.4)—Dissolve 27 g Trizma base and 13.75 g boric acid in $\sim$ 400 ml sterile distilled water. Add 10ml of 0.5MEDTA pH 8.0. Adjust the final volume to 500 ml with sterile distilled water. The pH should be 8.2–8.5 without further adjustment. Store at room temperature for up to one month. Discard if a precipitate is visible.

40%Acrylamide solution—Dissolve 95 g acrylamide and 5 g bisacrylamide, and adjust the final volume to 250 ml with distilled water. Filter and store at 4°C protected from light for up to several months.

10% (wt/vol) Ammonium persulfate—Dissolve 0.5 g of ammonium persulfate in sterile water, and then adjust the final volume to 5 ml. The solution may be stored at 4°C for several weeks. However, freshly prepared solution should be used to ensure effective gel polymerization.

5% (wt/vol) Acrylamide, 7.7 M urea sequencing gel—Mix 7.5 ml of 40% acrylamide solution, 27.72 g urea and 6 ml 5 $\times$ TBE, and adjust the final volume to 60 ml. Heat slightly and stir until all the urea has dissolved (make sure the solution cools down to room temperature before proceeding to the next step). Add 600 μl of 10% (wt/vol) ammonium persulfate and 36 μl TEMED, mix gently and promptly cast the gel. Allow the gel to solidify overnight at room temperature before running samples.

Diluted DIG-labeled DNA molecular weight marker solution—Mix 2 μ l of DIG-labeled DNA molecular weight marker stock (Roche), 14 μ l sterile water and 14 μ l sequencing gel-loading buffer. Store at -20°C for up to several weeks. Heat denature immediately before use.

Maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5)—Dissolve 11.61 g maleic acid and 8.77 g NaCl in $\sim$ 800 ml distilled water. Adjust pH to 7.5, initially with NaOH pellets, and then 10 N NaOH. Adjust the final volume to 1 l with distilled water, and then autoclave. The buffer may be stored at room temperature for several months.

10 $\times$ Blocking solution (10% (wt/vol) blocking reagent in maleic acid buffer)—Dissolve 5 g of blocking reagent in 40 ml of maleic acid buffer by gently heating it (do not allow the solution to boil). Once completely dissolved, adjust the final volume to 50 ml with maleic acid buffer. Store at 4°C for up to a month and dilute 1/10 in maleic acid buffer at the time of use.

Washing solution—0.03% (vol/vol) Tween 20 in maleic acid buffer. Prepare a fresh batch at the time of use.

Detection buffer (100 mM Tris, 100 mM NaCl, pH 9.5)—Dissolve 3.05 g Trizma base and 1.46 g NaCl in $\sim$ 200 ml distilled water. Adjust pH to 9.5, and the final volume to 250 ml, and autoclave. Store at room temperature for up to several months.

PROCEDURE

Isolation of short DNA-leading strands by alkaline gel electrophoresis ● TIMING $\sim$ 21 h

- 1| Dissolve 1.25 g of agarose in $\sim$ 90 ml of sterile water.
- 2| Cool down while stirring until the solution reaches $\sim$ 60 $^{\circ}\text{C}$.
- 3| Adjust the volume to 94.8 ml with sterile water.
- 4| Add 200 μ l of EDTA 0.5 M pH 8.0 and 5 ml of NaOH 10 N, in that order.
- 5| Mix well and quickly pour $\sim$ 80 ml of the gel mixture into a 14 cm $\times$ 10 cm gel-casting tray with a preparative comb.
- 6| While the gel solidifies, pretreat cells on a 10-cm culture dish for 1 h at 37°C with 2 μ M emetine (diluted in fresh, prewarmed growth medium).
- 7| Carefully dispose of the culture medium, and wash the cells (still on the plate) twice with 1 $\times$ PBS.
- 8| Trypsinize cells by adding 1 ml of trypsin-EDTA solution and incubating for 3 min at 37°C (or until cells have detached from the surface of the culture dish).
- 9| Add 4 ml of 1 $\times$ PBS, transfer the cell suspension to a 15-ml conical tube, and centrifuge at 500g for 5 min at room temperature.
- 10| Resuspend the cell pellet in 1 ml of 1 $\times$ PBS and transfer it to a 1.5 ml microcentrifuge tube.

- 11| Centrifuge the cells in a microcentrifuge at 400g for 5 min at 4 °C.
- 12| Resuspend the cell pellet in 260 µl of 1× PBS + 10% (vol/vol) glycerol solution containing 0.025% (wt/vol) bromophenol blue.
- 13| Load 85 µl of the cell suspension into one preparative well of the alkaline gel.
- 14| Add 25 µl of 5× SDS lysis buffer to each well and mix gently.
- 15| Incubate for 10 min at room temperature.
- 16| Add to each well 10 µl of 0.25 M EDTA pH 8.0 and 6.5 µl of NaOH 1 N (in that order). Mix gently each time.
- 17| In a separate well, load 30 µl of the 'DNA ladder mix'.
- 18| Add alkaline running buffer on both sides of the electrophoresis chamber until the solution reaches the top edge of the gel, without covering it.
- ▲ **CRITICAL STEP** Carry out electrophoresis initially under semi-dry conditions to prevent samples from floating out of the wells.
- 19| Run at 15 volts for ~2 h or until the bromophenol blue dye has completely entered the gel.
- 20| Add alkaline running buffer so that the entire gel is submerged, and then carry out electrophoresis overnight at 15 volts.
- 21| Neutralize the gel by incubating for 45 min in 1× TAE buffer.
- 22| Cut the gel to separate the size marker from the sample lanes.
- 23| Stain the gel containing the molecular size marker for 15 min in 1× TAE buffer containing 0.6 µg ml⁻¹ ethidium bromide. In the meantime, keep the rest of the gel (containing the nascent DNA) in 1× TAE (i.e., nonstained).
- 24| Visualize the stained gel under UV and, based on the migration of the ladder, cut out the portion of the nonstained gel containing 1–2 kb nascent strands from each sample lane.
- 25| Transfer the gel slices into a microcentrifuge tube, and extract nascent DNA molecules using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. Due to the limit of the gel amount that can be loaded into an individual extraction column (maximum 400 mg), it is typical to use three columns to extract the entire 1–2 kb fraction from a single agarose gel lane. Elute each column using 35 µl of sterile water and then pool the three elutes into one vial before proceeding to the next step.
- **PAUSE POINT** The nascent DNA can be stored for several weeks at –20 °C.

One-way PCR-based primer extension ● TIMING ~4 h

- 26| Set up PCRs in a final volume of 30 µl, which contains (final concentrations): 1× magnesium-free PCR buffer, 3 mM MgSO₄, 400 nM digoxigenin-labeled primer, 200 µM each dNTP, 1 M betaine, 1 U of IDPol DNA polymerase and 5 µl of the nascent

DNA preparation (use 5 μ l of sterile water for no-template control). We used an equal volume of nascent DNA when analyzing the same DNA preparation with different primers. If comparing different DNA preparations, we used an equal amount of nascent DNA, which we determine by quantitative PCR analysis.

▲ **CRITICAL STEP** For RIP mapping at the *DBF4* locus, the addition of betaine is crucial for the success of one-way PCRs. This is probably because the *DBF4* ori is very G+C rich. However, one should consider omitting betaine if the DNA sequence is A+T rich.

? TROUBLESHOOTING

27| Centrifuge the tube briefly to collect all the reaction solution at the bottom, and then carry out PCR as follows: initial incubation for 5 min at 95 °C, 30 cycles of amplification (1 min at 94 °C; 1 min at the appropriate annealing temperature (usually 68 °C) and 1.5 min at 72 °C), and final extension for 7 min at 72 °C, followed by incubation for 10 min at 4 °C.

28| Add 20 μ l of sequencing gel-loading buffer to each tube.

29| Heat the samples for 4 min at 90 °C, and quickly chill on ice.

■ **PAUSE POINT** Samples can be stored for several months at –20 °C. If this is the case, they should be re-denatured by heat treatment (Step 29) at the time of use.

Sequencing gel fractionation and digoxigenin detection ● TIMING ~20 h

30| Separate the primer extension products (2 μ l) by electrophoresis on a 5% (wt/vol) acrylamide gel prepared in 0.5 $\times$ TBE and containing 7.7 M urea. Run the gel for ~2 h (or until the bromophenol blue has migrated ~20 cm from the top of gel) at 5 watts, using freshly prepared running buffer (0.5 $\times$ TBE). In the same gel, run 2 μ l of ‘Diluted DIG-labeled DNA molecular weight marker’.

31| Transfer DNA from the gel onto a Hybond N+ membrane using a semi-dry transferring apparatus for 1 h at 400 mA with 0.5 $\times$ TBE. Due to the limited dimensions of the commercially available transferring apparatus, only a portion of the gel may be transferred at a given time. This gel portion usually cannot be larger than 20-cm long and 10-cm wide. Despite this size limit, the 20 $\times$ 10 cm dimension is usually sufficient to detect all the digoxigenin-labeled (molecular weight) DNA bands at relatively good resolution. However, if higher resolution is desired, one may load two or more sets of samples on the same gel at different times. Different portions of gel (e.g., “top” and “bottom”) can then be transferred separately onto membranes. Carefully remove the glass plate that is not attached to the gel, and then place a pre-cut piece of thin chromatography paper on top of the gel portion from which DNA will be transferred onto a membrane (note that the gel is still attached to the other glass plate). Carefully cut the gel around the contour of the paper to separate the sample-containing gel portion from the remainder of the gel. Grab the paper (with the gel still attached to it) by a corner, and slowly and carefully peel it off the glass plate. Note that the paper is still attached to the gel while DNA is transferred onto a Hybond N+ membrane that is placed on the opposite side of the gel.

32| Fix the membrane for 6 min at 302 nm wave length (i.e., 'high' setting on a TM-36 UV Chromato-Vue Transilluminator).

33| To reduce background, 'block' the membrane for 1 h at room temperature using freshly diluted 1× blocking solution in maleic acid buffer.

34| Incubate the membrane overnight at 4 °C in 1× blocking solution containing a 1/10,000 dilution of the antidigoxigenin antibody. To minimize the amount of antibody, use a heat-sealed plastic bag to carry out this incubation step.

35| Wash the membrane twice for 15 min each with freshly prepared washing buffer at room temperature.

36| Incubate the membrane for 1 min in detection buffer.

37| Place the membrane in a new heat-sealed plastic bag and cover with CDP-*Star* substrate diluted 1/100 in detection buffer. Incubate for 5 min at room temperature.

▲ CRITICAL STEP To prevent contamination, the vial of CDP-*Star* substrate should be opened only under a sterile environment.

38| Remove excessive solution from the membrane, and then expose it to a film. The approximate size of one-way PCR-based primer extension products can be determined by comparing the position of a sample band and those of the digoxigenin-labeled molecular weight markers. Subsequently, a RIP position can be estimated by combining the primer position and the band size determined by gel electrophoresis¹⁰.

? TROUBLESHOOTING

● TIMING

Steps 1–25, 21 h (including overnight gel electrophoresis)

Steps 26–29, 4 h

Steps 30–38, 20 h (including overnight antibody incubation)

? TROUBLESHOOTING—Troubleshooting advice can be found in Table 2.

ANTICIPATED RESULTS

Figure 1a shows a typical result of RIP mapping at the *DBF4* ori using the protocol presented here. The size of the top band shown in Figure 1a (lane '–') is estimated to be 504 nucleotides. Since the start site of primer pC is –131 (i.e., 131 nucleotide upstream from the ATG translation start site), the RIP of this leading strand was determined to be the +373 position (Fig. 1b). This position was later confirmed by sequencing gel electrophoresis¹⁰. As expected, no bands were observed in the control sample lacking template DNA (Fig. 1a, lane 'N'). Similarly, primer extension signal was greatly reduced when the nascent template DNA was isolated from aphidicolin-treated cells (Fig. 1a, lane '+').

By carrying out detailed RIP mapping with many different primers, we discovered that *DBF4* ori contains two replication initiation zones that are regulated by an asymmetric

bidirectional replication mechanism¹⁰. This finding was possible because we could construct a fine RIP map at this locus using the one-way PCR-based primer extension method described here. It should be noted that a nascent strand abundance assay alone, which is commonly used for mapping replication ori, could not detect two replication initiation zones¹⁰. Therefore, the protocol presented here is a very powerful tool in unraveling the regulation of replication oris.

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Figure 1.

RIP mapping of the human *DBF4* ori by one-way PCR-based primer extension. **(a)** Primer extension with primer pC (position is shown in **b**) identified two RIPs for 'antisense' (i.e., the opposite direction of *DBF4* transcription) leading strands emanating from the *DBF4* ori locus. The reaction was carried out with 1–2 kb nascent DNA fragments isolated from HeLa cells maintained for 1 h post-G₁/S arrest in the presence of emetine (lane '–'). The control cells were treated in the same manner, except 5 $\mu\text{g ml}^{-1}$ of aphidicolin was also included along with emetine (lane '+'). L, digoxigenin-labeled molecular weight ladder (sizes in nucleotides are shown to the left of the panel); N, no template control. Numbers in brackets to the right of the panel indicate the approximate positions of RIPs determined based on band sizes. Note that the '–' and '+' before numbers denote, respectively, the upstream and downstream positions of the *DBF4* ATG codon. **(b)** A physical map of the human *DBF4* locus. The position of primer pC is shown. The position '–131' denotes 131 nucleotides upstream of the ATG translational start codon ('A' of the ATG is +1). The +100 denotes 100 nucleotides downstream from the ATG start codon. The vertical dashed lines are leading strand start sites at the *DBF4* ori. Arrows are the direction of replication deduced from data shown in the panel **a**.

TABLE 1

Summary and comparison of different approaches in determining RIPs.

Steps	Gerbi and Bielinsky ^a	LM-PCR ^b	This protocol ^c
Nascent DNA isolation, purification	<ul style="list-style-type: none"> • Genomic DNA extraction by CsCl centrifugation or DNAzol • BND chromatography • T4 polynucleotide kinase treatment • λ-exonuclease digestion 	<ul style="list-style-type: none"> • Phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation • Sucrose gradient centrifugation • T4 polynucleotide kinase treatment • λ-exonuclease digestion 	<ul style="list-style-type: none"> • In-gel cell lysis and alkaline gel electrophoresis • Gel extraction
Primer extension	<ul style="list-style-type: none"> • 30 cycles of one-way PCR amplification using radioactive-labeled oligonucleotides and Vent (exo⁻) DNA polymerase 	<ul style="list-style-type: none"> • Second strand DNA synthesis • Ligation to double-stranded asymmetric linkers and PCR amplification (LM-PCR) • Radioactive primer extension using Vent (exo⁻) DNA polymerase 	<ul style="list-style-type: none"> • 30 cycles of one-way PCR amplification using nonradioactive primers and IDPol DNA polymerase
Sequencing gel electrophoresis and detection ^d			

^aThe protocol described by Gerbi and Bielinsky² is as follows: bulk genomic DNA is isolated using CsCl gradient centrifugation or DNAzol extraction², followed by BND chromatography, T4 kinase treatment and λ-exonuclease digestion to enrich nascent DNA. 5'-end mapping of the nascent strands is achieved by 30 cycles of standard one-way PCR amplification.

^bThe method used by Abdurashidova and colleagues to map the RIPs at the human lamin B2 ori¹. This protocol incorporates an LM-PCR step to increase sensitivity.

^cThe modified one-way PCR-based RIP-mapping procedure described in this protocol was used to map the RIPs at the human *DBF4* locus¹⁰.

^dAll three RIP-mapping protocols include the analysis of amplified DNA by sequencing gel electrophoresis.

TABLE 2

Troubleshooting table.

Problems	Possible reasons	Solution
Spotted background observed on the membrane (Step 38)	Precipitation of antidigoxigenin antibodies in the vial	Quick spin the antibody vial immediately before use
No signal in any lanes, including molecular weight marker (Step 38)	DNA transfer to the membrane was not successful	Ensure that the DNA transfer equipment is working properly
	DNA was not properly fixed to the membrane after transfer	Fix with UV immediately after transfer to a membrane
No signal in one-way PCR samples (Steps 26, 38)	PCR conditions may be too stringent	Eliminate/reduce betaine from the PCRs and/or reduce the annealing temperature
Double bands observed in molecular weight ladder (Step 38)	Incomplete sample denaturation	Heat samples for at least 4 min at 90 °C and quickly chill on ice before gel electrophoresis